Crohn's Disease – Is There a Microbial Etiology?
Recommendations for a Research Agenda

December 14, 1998
Bethesda, Md.

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Sponsored by: NIAID, NIDDK
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Goals of the Workshop

This conference was held in the Natcher Conference Center on the NIH campus in Bethesda, Maryland on December 14th, 1998. The purpose of the conference was to review the current state of knowledge relevant to a microbial etiology of Crohn's disease (CD), a serious, debilitating, inflammatory bowel disease. In particular, we set out to review evidence for and against the hypothesis that the bacterium, *Mycobacterium avium* subspecies *paratuberculosis* (Map) is the

cause of CD, and to define needed research that could shed light on the etiology and pathogenesis of this chronic disease.

One of the major objectives of the conference was to have a productive scientific exchange between researchers with differing views on whether Map is a human pathogen and if it is involved in the pathology of CD. There has been little agreement on this point in the scientific community. Much exciting research conducted in the 1980's on this organism failed to provide conclusive evidence that it was the cause of CD. The difficulty stems from the fact that the organism is extremely difficult to grow in the laboratory and to detect or recover from patients. Additionally, it has been difficult to demonstrate an immune response to Map in patients with CD. In spite of this, there exist a number of respected clinicians who treat Crohn's patients with antibiotics effective against mycobacteria, and who believe that there is an improvement in their condition. Moreover, a number of patients and their advocates support the use of antibiotics, and gave personal testimony at the conference to the improvement of their disease symptoms following such treatment. They advocate the conduct of controlled antibiotic treatment trials to resolve this issue. A major objective of the workshop was to define new research opportunities and to explore possible mechanisms for stimulating additional research.

Conclusions

- There is insufficient evidence to prove or disprove that Map is a human pathogen or that it is the cause of Crohn's disease. Considerable controversy continues to exist in the scientific community on this point.
- The difficulty in detecting and growing the organism, or being able to demonstrate a consistent immune response to it in CD patients, continues to frustrate researchers.
- Newer methodologies including the detection of microbial DNA sequences and functional genomics are available and should be applied in the search for a microbial etiology of Crohn's disease. A wide net should be cast. Characterization of microbial "communities" associated with this disease should be sought. Comparison with the completed genome sequences of the related species *M. tuberculosis, M. leprae*, and *M. avium,* may provide clues to the genetic basis of the pathogenesis of Map.
- Good animal models for CD do not exist and should be sought. The development of additional genetically engineered mouse strains or primate models may be worthwhile, since current models do not develop much of the pathology associated with CD. Large animals, including sheep and cattle that get Johne's disease from Map, should be treated with vigorous antimicrobial, anti-inflammatory, and immunosuppressive treatment (typical of what is used in humans) in conjunction with molecular characterization of associated microbial populations and strains.
- If Map is proven to be a human pathogen, there is the potential for an enormous impact on human health due to the prevalence of this organism on the farm and in water. Further study of Map as a food and/or waterborne pathogen should be conducted. Viable Map should be sought in commercial milk and other dairy products as well as in meat. Conclusive studies of the effectiveness of pasteurization using commercial equipment and process rather than laboratory simulations should be performed. In order to conduct the above, standard methods for the concentration, detection, and *in vitro* culture of Map should be developed and used by participating researchers. Federal agencies with regulatory authority over the food supply should consider conducting such research in cooperation with relevant food production industries and academic researchers.

Research Recommendations

Basic and clinical research should be aimed at answering the following fundamental question: Does Map, or other microbial pathogen(s), cause CD? Answering this question requires addressing the following additional questions: Do affected tissue samples from Crohn's patients consistently contain Map or any other pathogen? Can we detect specific immune reactions to a CD associated pathogen? What is such a pathogen's phenotype and genotype? Can we make the disease better by using appropriate antimicrobial drugs?

Workshop participants identified the following specific research needs.

Clinical Studies:

1. **Determine potential infectious etiologies of CD** by collecting and studying biopsy tissues from the intestines of Crohn's patients (stratified into perforated and contained lesions) and controls, and using sensitive diagnostic methods to enumerate any microbial flora associated with the disease. The use of anti-inflammatory drugs before obtaining biopsies may serve to close lesions/ulcers so that there is less contamination with normal gut flora or foodborne organisms. Ribosomal RNA typing (ribotyping) and other newer methodologies (such as subtractive hybridization) should be applied to tissues, as well as more traditional microbial culture and diagnostic techniques. Patients should be clinically well defined in terms of stage (quiescent or active) and duration (recent or long term) of disease, and tissues should be collected under defined standardized protocols. Such a search should not look exclusively for Map, but should cast a wide net, seeking perhaps a "suite of organisms".
2. **Define the host immune response in Crohn's Disease.** What are the factors that contribute to the continuing inflammatory cascade observed in Crohn's disease? Normal flora, pathogens, diet, and stress have all been suggested as contributors to disease. Is initial infection with Map or another organism acting to "prime" the immune system to respond to other stimuli in an abnormal, pathologic way? Will elimination of an underlying chronic infection allow the immune system to behave more normally? Immune cells in CD and control biopsy tissues should be analyzed and compared. If there is a microbial etiology, definition of the antimicrobial immune response will be important.
3. **Conduct epidemiological research** to elucidate risk factors for human infection. Studies of farm workers and their families should be performed using modern diagnostic methods. Evidence of occupational or farm-life exposure to domestic animals should be sought in recently "emergent" clusters of CD. Studies should include prospective surveillance of young children to see if and when they may be infected with Map (seroconversion to P35 and P36 or other antigens). Clinical specimens, if obtained, should be probed for the IS900 repetitive element or any other repetitive element identified in Map. Evidence should be sought for the presence of Map in dairy products, meat, and domestic water sources.
4. **Conduct genetic studies** of families with a history of CD. Linkages have been tentatively assigned to chromosomes 1P, 4Q, 3 and 16, and 12. Are there others? What are these loci? What are the genes and what role do they play in CD? If a better animal model of CD were available, such genetic analysis might be facilitated.

Antimicrobial treatment of CD. The use of anti-mycobacterial chemotherapy in the context of Crohn's disease is controversial. Many clinical studies employing empirical antimicrobial chemotherapy have been performed and investigators have reached different conclusions regarding the role of Map in CD. NIH is supportive of finding resolution of this issue and would welcome the opportunity to work with clinical investigators on case definition, experimental design, and tissue collection protocols that would permit meaningful molecular and microbiological studies as part of future antimicrobial treatment protocols. NIH-supported

investigators and available laboratory facilities may be helpful and could provide expertise and support in the conduct of studies to determine if there is a microbial etiology of CD. Some of the approaches that should be taken are described elsewhere in this document and may be conducted as part of future clinical strategies not requiring definitive blinded trials. Recommendations for study design of treatment protocols include 1) CD case definition should be developed by participating investigators with the help of NIH and should be consistently applied in various clinical protocols. 2) Cases should be stratified into aggressive (perforating) and contained (non-perforating) pathology as well as to stage (active or quiescent) and duration of disease. 3) If Map is the target of antimycobacterial therapy, minimal inhibitory concentrations (MIC's) of the antibiotics proposed for use should be determined prior to start of the trial employing clinical isolates of Map (as opposed to lab strains) to insure that effective therapy is delivered. 4) PCR and serology pre-, during and post-treatment in conjunction with culture studies should determine Map status. 5) Follow-up should be planned to determine the incidence of reinfection or disease recurrence. 6) Clinical specimens should be obtained which would be suitable for ribotyping, PCR, subtractive hybridization, or other sensitive methods as discussed elsewhere in this report. Properly obtained samples will be invaluable for the purpose of defining the microbial flora associated with CD lesions. For this reason, consent documents should indicate that tissue samples and sera will be stored and used for research purposes. Because evidence linking Map to CD is not conclusive, the conduct of large, multi-center, blinded, placebo controlled trials of anti-mycobacterial drug therapy may be premature at this time. Such treatment protocols are complicated by the lack of sensitive and specific diagnostic tests for Map and the difficulty in culturing the organism from clinical specimens, making stratification of cases based on Map status difficult. When evidence is available to better support this, or any other microbial etiology, blinded antimicrobial trials of appropriate drugs at effective doses should be considered. Such evidence can be obtained by cooperative efforts between clinicians and basic scientists, and NIH can assist in this effort.

Basic Studies:

If Map is established as a likely etiologic agent by clinical studies (see #1 above), basic investigations of Map pathogenesis should be performed. If another pathogen(s) is/are identified as playing a role in CD pathogenesis, similar studies of such pathogen(s) should be performed.

1. **Establish cell or organ culture models** of infection focusing on growth characteristics and gene expression of Map in cell culture (ex. macrophages, intestinal epithelial cells). Does *ex vivo* growth of Map (in organ or cell culture, for example) affect pathogenicity in an animal model?
2. **Establish new animal models of Map infection**. Clinical isolates of Map should be used. A small animal model would be ideal, but has been elusive. Genetically engineered knock out mice or rats may be useful. Primate models may be helpful, but would be costly. Characterize the virulence and host preference of different Map strains obtained from humans or animals. Determine the minimal infectious dose for Map in an animal. Determine whether the infectious dose varies in animals of different ages.
3. **Develop an improved large animal model of CD.** Treat animals with Johne's disease for extended periods with antibiotics and/or immunosuppressive drugs (including thalidomide?) in an attempt to develop a better animal model of Crohn's disease and to see if Johne's disease can be cured (long term follow-up). Determine the effect of such treatment on inflammation and on the levels of cytokines and other immunomodulators.
4. **Perform in vivo expression technology (IVET) studies** in animals susceptible to Johne's disease to identify bacterial genes uniquely expressed *in vivo*. Such studies may be instructive of what to look for in other animal models and might provide valuable new information on the importance and role of new virulence factors in human disease.

5. **Compare Map DNA sequences** to available genome sequences of other mycobacteria. These comparisons may yield clues to pathogenicity. Is there a role for genetic insertion elements such as the Map IS900 in pathogenesis? Gene expression arrays developed for other sequenced mycobacteria may be useful in determining if there are analogous virulence genes expressed in Map, for example. Are there genes in Map or other mycobacteria that may be homologous to virulence genes in other intracellular pathogens (*Salmonella, Shigella, Listeria,* and *Chlamydia* for example).
6. **Identify and optimize diagnostic Map antigens** that can be isolated or produced by recombinant technology or other means and made widely available to researchers. Purified peptide, carbohydrate, and lipid epitopes should be sought.
7. **Adapt antibiotic susceptibility testing methods** to deal with a species that grows even more slowly than the so-called "slow growing mycobacterial pathogens". Studies should be expanded to look at combinations of drugs and to look at their efficacy against intracellular organisms and spheroplast forms. Drugs that are effective *in vitro* should be examined for efficacy against Map infection in cell culture, in animals and eventually in humans.
8. **Determine the relationship** between Map and the *M. avium* complex, whether from Crohn's disease or Johne's disease. Molecular techniques including ribotyping, multi-locus enzyme electrophoresis, and DNA fingerprinting could be used to characterize and distinguish species. The Map specific IS900 element has proven valuable in this regard. Comparative difference sequencing could identify other candidate markers and may lead to more useful diagnostic reagents and methods.
9. **Develop a high-density array of ribosomal DNA or RNA** on a chip that can be used to more completely define the organisms associated with Crohn's disease. Use such a chip to examine tissues from patients with differing disease severity and duration.
10. **Apply subtractive hybridization techniques** to look at the difference between CD tissues obtained by intestinal biopsy, tissues from a non-involved area of the intestine from the same CD patient, and normal tissues from controls. Tissues from early apthous or focal lesions as seen in post-operative recurrence models or in areas adjacent to grossly involved areas should be studied.

List of Presenters

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Dr. Michael Collins University of Wisconsin Madison, WI	Dr. R. Balfour Sartor University of North Carolina Chapel Hill, NC
Dr. Fouad El-Zaatari Baylor College of Medicine Houston, TX	Dr. David Schauer Massachusetts Institute of Technology Cambridge, MA
Dr. Robert Fleishman The Institute for Genomic Research Rockville, MD	Dr. Herbert van Kruiningen University of Connecticut Storrs, CT
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Advisory Panel

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Edited Transcript of the Workshop

Dr. Patrick Brennan: Introduction

I think this is a very opportune time to hold this meeting. Thanks to NIAID and NIDDK for sponsoring the meeting. For those of us who have been involved in this research area in the past, this is a glorious opportunity to get together and reconsider this important disease. Previously I've been involved with some of the present researchers in the 1980's and this was a time of great opportunity and great ferment in the area. It was a time where we went from considerable doubt, considerable worry, and considerable controversy regarding the etiology of CD and began to focus on the possible relationship to infection with Map. At that time there was very interesting bacteriology, the discovery of mycobacterial spheroplasts, and the development of new probes reflective of repetitive sequences in the genome of Map. There was much activity, there was much sharing of resources, there was much research on the development of a serodiagnostic procedure. Then there was almost a stoppage of research towards the end of the 1980s. Little has happened at the basic research level in the intervening years.

I hope that we have a good exposition, a good analysis of the data available on the whole issue of a possible etiology for Crohn's disease, whether it's mycobacteria, what type of mycobacteria, and how it might be related to *M. paratuberculosis*. Then out of this discussion, we hope to be able to analyze the information and make solid recommendations to NIH on possible research opportunities, and research directions. I think this is our mission and it would be delightful if we could stay focused on this mission. This is probably the most important thing that we can do, the most important contribution that we, as fundamental scientists and a scientific community, can make to this disease.

Probably my most demanding task is to try and assure that everybody has the opportunity to present their opinions, to present their data, and that there is good discussion taking place on that data. With that, we should proceed to the scientific presentations and scientific discussions. I call upon Dr. Bayless.

Theodore M. Bayless, M.D. - Crohn's Disease as a Clinico-Pathologic Entity

CD is one of the two idiopathic inflammatory bowel diseases; the causes of which are unknown. CD is a chronic illness that has flare-ups and remissions. It affects young people, the average age of onset being 27. Although CD has increased in prevalence over the last 40 years in the United States and throughout most of the countries in Europe and Scandinavia, the illness itself hasn't changed. The average age has been virtually unchanged in these 40 years. The location of inflammation is in the ileum in 35% of patients. That has not changed, nor has the 35% of patients that have granulomas in the small bowel or colon.

In young people, the earliest lesions in the intestine are areas of focal inflammation. If one were to look for a microbial or viral cause of CD, these early lesions would be the place to start. Smoking is known to increase the severity of CD, whether it is a risk factor for susceptibility, we don't know. The influence of diet on the disease is not known. The pathogenesis of CD clearly involves the immune system; one has to have T4 cells to have active CD. Steven James showed that when a person develops AIDS and their T4 levels fall, CD becomes quiescent. In five or six patients recently reported with allogeneic bone marrow transplants, who received intensive immunosuppression before the transplant, CD either remitted or stayed in remission. That may be an argument against the involvement of a latent, chronic, microbial infection that would be expected to increase in severity in immunosuppressed individuals. Sixty percent of his CD patients are being treated with azathioprine or 6-mercaptopurine with a positive response seen in about 70% of those treated.

The pathogenesis of CD may also involve the regular gut flora. Parenteral feedings or elemental diets essentially starve the colonic bacteria and CD symptoms improve.

There is also a genetic predisposition to develop CD, 30% of young patients have a positive family history of CD. CD occurs more frequently in people of European descent, in whites, and in Ashkenazi Jews. They are at about four or five times greater risk than other populations. If a person has CD, their relatives are at ten-fold greater risk than the general population. In homozygous twins, at least 50% are concordant for CD. If both parents have inflammatory bowel disease, 50% of their offspring develop CD by the age of 20. Ten research groups around the world are looking for genes that may be linked to CD susceptibility. Linkages on chromosomes 1P, 4Q, 3, 16, and 12 have been found. This probably will not be a simple case of one gene being responsible. It is likely to be a very complex situation. Whether any of the genes present in families will be found to play a role in sporadic cases remains to be seen.

In terms of epidemiology, there is a lack of increased incidence in the spouses of patients, of physicians treating CD. This observation does not support the notion of an infectious agent causing CD. On the other hand, there is a single report of a technician working with *Mycobacterium* who got CD.

Dr. Bayless then outlined a proposed model for CD. The antigen, whatever it is, viral, bacterial, or dietary gets across the intestinal barrier but the normal suppressive immune response does not occur. There is no activation of suppressor T cells and tolerance to this antigen does not develop. For example, tolerance would be expected if one eats strawberries or oysters or almonds for the first time.

In CD there appears to be antigen recognition and an increase in activation of helper T4 cells, which leads to a Th1 response, inducing a variety of cytokines and inflammatory mediators that lead to inflammation. There has been a case report of a patient with CD who had a small bowel transplant, and the disease recurred in the transplanted bowel suggesting that the immune cells carried the message to that new segment of intestine. Remissions with allogeneic bone marrow transplantation were mentioned earlier.

Environmental factors also play a role: children who are exposed to poor sanitation seem to be somewhat protected from CD and the disease is more common in urban areas. As mentioned, intestinal contents seem to be a major factor in exacerbating CD. The mechanisms seem to be an exaggerated and prolonged immune inflammatory response with the T4 cell being at the center of many of these mechanisms. CD occurs in North America, Europe, Scandinavia, and areas where Europeans have moved. It is now appearing in Japan and Asia.

Anti-inflammatory therapy works in about 50-60% of moderately ill patients. A variety of agents such as 5-aminosalicylate products and prednisone are helpful. Alteration of luminal contents can help. Bowel rest can be helpful, or antibiotics, which seem to alter the intestinal flora; metronidazole and ciprofloxicin being those most commonly used. Clarithromycin, an antibiotic effective against some mycobacteria, has been used in one study showing a 50% response rate. If those treatments are not working, one alters activated T cells using immunomodulators: azathioprine, 6-mercaptopurine, cyclosporin, and more recently an antibody to TNF has been developed (Remicade) which seemingly is helpful in suppressing inflammation in CD. The point is that immunosuppression that is utilized for CD does not exacerbate the disease as one might expect if a *Mycobacterium* was the causative agent.

Importantly, there has been no evidence of cellular of humoral immunity to *Mycobacterium* and since we think the immune response is really the key to keeping this illness going, one would anticipate that if *Mycobacterium* was involved that one would have a strong cellular or humoral response to that organism.

What is the role of infectious agents in CD? In terms of etiology, we don’t know if it's viral or bacterial. Infectious agents, especially adenovirus can be the activating triggers in people with ulcerative colitis. Also, organisms such as cytomegalovirus can be aggravating factors in people with established IBD who are immunocompromised. *Clostridium difficile* can also cause diarrhea in a patient with Crohn's disease; luminal bacteria are important in perpetuating active inflammation. Dr. Sartor will discuss the role of gut flora in keeping Crohn's active. When you resect areas of CD, as you do in 50-60% of patients, the illness returns in half the patients. Dr. Bayless believes that fecal bacteria play a role in the site of recurrence as well as in perpetuating inflammation.

Why might antibiotics have been shown to work in some CD patients? They might eradicate bacterial antigen triggers, which are probably an important factor in pathogenesis. They might eliminate bacterial overgrowth due to the stenoses that occur in CD. They might reduce the pro-inflammatory bacterial toxins, and some of them, such as metronidazole and ciprofloxacin have immunosuppressive properties, as does Dapsone, which has been used to treat some of the patients with CD who were getting anti-mycobacterial therapy.

The CD intestine, at its worst resembles a cobblestone street. Due to ulcerations, the mucosa may become permeable to luminal antigens. The small intestine might be permeable to dietary food antigens, including mycobacteria if present in food. Various antigens do cross the altered small intestine. There has been recent interest in *Saccharomyces cerevisiae*, the yeast used in fermenting beer. Fifty-sixty percent of patients with CD have antibodies to this dietary food antigen. One would anticipate that such antigens might get through the small intestine. They would perhaps eventually be destroyed by digestion, so they might not be as common in patients who have damage to the colon, as in ulcerative colitis where only 10% of the patients who have a damaged colon had antibodies. One wonders whether the evidence suggesting that a *Mycobacterium* has been in the intestine, might be a reflection of its occurrence in food that could cross a damaged mucosa.

The early lesions of CD are focal or are apthous ulcers. Granulomas are not seen in this part of the lesion, a point against a mycobacterial etiology. These are areas where the mucosal

permeability is altered, and if Map were the cause of Crohn's disease one would like to be able to find the organism (or any other) in these early lesions.

As the pathology of CD progresses, fistulizing or perforating disease occurs. This is not a feature of Johne's disease of cattle. The deep fissure is unique for CD. Also, after 8-10 years of illness, a fibro-stenotic obstruction develops. Of the 11-15 cases of reported culture of Map from Crohn's patients, most of those had surgery and had the illness for a long time and received a variety of anti-inflammatory, immunosuppressant, and antibacterial agents. Only one-third of the patients with Crohn's have granulomas, while granulomas are universal in Johne's disease. The granulomas in CD are different. These are not caseating. The granulomas do not have anything to do with the activity of the disease, nor with the recurrence of CD.

The gut has a very limited repertoire of response to pathologic injury. CD, with its grossly thickened wall resembles tuberculosis, but one finds acid fast organisms in tuberculosis. This obviously allows one to make a diagnosis. Johne's disease has gross similarities to CD. As mentioned, the granuloma, which is so important in Johne’s disease, is only found in one third of patients with CD.

In sum, Dr. Bayless does not believe that there is an association of Map with CD. He thinks the lack of consistent identification in tissues and the lack of systemic and mucosal cell immune responses argue against a mycobacterial etiology. Further, he believes that a lack of disease worsening with immunosuppression and a lack of epidemiologic support make it unlikely that Map is pathogenically associated with Crohn's. However, he feels that in some patients, MAP may be a factor in the development of inflammation. This would fit with the heterogeneous nature of CD.

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Question: Is there a clinical experience with bone marrow transplants for whatever reason in patients with Crohn's disease, and has CD been shown to remit in patients who undergo bone marrow transplants?

Answer: A recent paper in the April issue of Gastroenterology described six patients who had undergone allogeneic bone marrow transplant for histologic malignancies who had CD. In two, the Crohn's improved rather dramatically. In the other four, they stayed in remission. In none, did the illness worsen with intense immunosuppression as you might anticipate, and it was thought that perhaps you were producing or transferring another stem cell. One of the patients, interestingly, did not get a complete take of their marrow and the host cells persisted, and that person had a relapse of the Crohn's disease.

Question: What is the prevalence of CD in the United States?

Answer: The best estimates currently from epidemiologists are that there are four hundred thousand people in the United States with Crohn's disease, and perhaps 400,000 with ulcerative colitis. Various charitable associations have used larger numbers.

Question: Have antibodies to MAP been found in CD patients?

Answer: There's been no consistent documentation of a cell mediated immune response to Map. Yes, antibodies have been described but that probably is not the mechanism of the disease. It is quite clear that in Crohn's disease, there is a hyperactivity of the humoral immune response to many antigens including Baker's yeast; *E. coli* and other bacteria. A whole host of humoral immune responses to a host of environmental agents are involved. If this is an environmental agent present in the lumen, it's quite rational that the ulcers/mucosa would have increased uptake and then a humoral immune response would occur.

Question: I'm interested in the frequency of detection of granulomas in Crohn's disease. Is there a distribution of granuloma through the bowel wall? In other words, might granuloma be more likely to be found closer to the serosal surface than the luminal surface? If so, could the number that you cited, the one-third of patients, reflect the means by which tissue is sampled in most cases, via endoscope and mucosal?

Answer: It's true, there are more granulomas deep in the mucosa and the sub-mucosa. However, the number of one-third having granulomas is in resected specimens of the entire gut wall. The granulomas do not reflect the tendency to recur after surgery, nor are they found in these early apthous lesions.

Question: As you quite rightly pointed out that the number of granulomas we see in clinical practice is much less than what would be expected if the etiology was mycobacterial. But is it also true that most of the surgical specimens come from the patients who had been treated with immunosuppressive drugs, including steroids?

Answer: They do currently, but that information on granulomas in only 30% goes back 40-50 years. As I said, the illness hasn't really changed according to most observers. So, before immunomodulators were used, those same findings were present. When the illness recurs after surgery on no therapy, one would anticipate then if the granuloma was more important, then one would see granulomas early on in recurrence and one does not.

Dr. Michael Collins - Johne's Disease and Its Similarities to Crohn's

Dr. Collins made the following initial observations:

- The potential for Map to be pathogenic for humans is clear. The organism has a broad host range: infections have been documented in widely diverse animal species, including non-human primates.
- The probability that humans are exposed to Map is relatively high. It is important to understand that this is an obligate parasite. Everything we know about the biology of this organism indicates that it can replicate only in infected animals. Ruminants are the preferred host of Map and where most replication occurs. It may persist in the environment. Therefore, exposure of humans results from direct or indirect contact with products of animal origin.
- If Map infections present a human health problem, the magnitude of the problem is enormous because of the high rate of the infection in animal populations. It is continuing to spread among animals as we are doing little to curb the spread of infection.
- The need for research on Map as a potential human health problem is unquestioned. While we have done a tremendous amount of work on *M. avium*, we have done very little with Map that may be far more virulent.

Johne's disease was first described in the United States in 1908, coincidentally about the same time that Crohn's disease was first described. Consuming Map via the fecal-oral route can infect calves. Feeding milk can also infect them because infected cattle excrete Map in their colostrum and milk long before they show any clinical manifestations of the disease. The infection begins at Peyer's patches where the organism is taken up, much like many other gut pathogens, and is phagocytized by macrophages. In this environment the organism grows happily. The pH optimum for Map growth is close to that of a phagolysosome, pH 5.5 to 6. The reason we probably find this disease attacking the terminal ileum in the ruminant is that this is where the highest numbers of Peyer’s patches reside and they are extremely active in these very young ruminants.

This disease is not just a disease of dairy cows. Johne's disease also has been documented in several other species including sheep, pigs, horses, goats, deer, elk, and bison. Primates, including macaques, baboons, cotton top tamarins, and Gibbons have also been shown to get Johne's disease; this organism has a far broader host range than we have recognized in the past.

Typically, we expect to find abundant acid-fast organisms on histopathology with a diffuse non-caseating granulomatous inflammatory response to infection. However, there is a wide spectrum of pathology observed among infected animals both within and between species and sometimes we see very few organisms and very limited amounts of pathology. Many times pathologists look quite hard to find acid-fast organisms in animals with Map. It is not uncommon for pathologists to report that they can see cellular changes indicative of inflammatory changes typical of Johne's disease but see no acid-fast organisms. In such cases, only by culture or PCR can infection be documented. Multibacillary and paucibacillary forms of Johne's disease are well known and parallels have been drawn to the similar spectrum of pathology seen in leprosy. Failure to easily observe acid-fast bacteria in CD patients should not be given undue weight in considering the possibility that the disease is induced by mycobacteria.

A recent USDA survey indicates that 41% of U.S. dairy herds have at least one Map ELISA positive animal. Roughly 22% of all U.S. dairy herds have a 10% or higher infection rate. The infection rate is highest in larger dairy herds: 40% of U.S. dairy herds having 300 or more cows have a 10% infection rate. In contrast, among small dairy herds of 50 or fewer cows only 19% of herds have a 10% or higher infection rate. The infection is continuing to spread. Every time animals are moved, new herds become infected, and if nothing changes with regard to a Map control program in the U.S., the dairy herd infection rate may reach 100%. The vast majority of Map infections in any given animal population is subclinical. In all likelihood, 5-10 times as many asymptomatic animals are present than those showing outward signs of disease. It is important to note that these subclinical cases are infectious, shedding organisms in their manure and milk, and infecting other animals in the herd. The long prepatent period characteristic of this infection increases the challenge of detecting animals in the early stages of infection.

In the latter phases of the infection, the organism may become disseminated to other parts of the body of the animal. Although the bacteria can be isolated from many different tissues and organs, a granulomatous tissue response in these other body sites is not commonly seen, except in the liver. This probably indicates that dissemination of Map infection from the gut is a late event in the pathogenesis of Johne's, and the animal does not have sufficient time to mount a cellular immune response in those locations. The liver granulomas are notably common in goats. This disseminated nature of Map has profound implications for the food chain.

There are now nine different laboratory tests for diagnosis of Johne's disease, and five of these are available as commercial kits. These tests are based on finding Map, or detecting an antibody response or cellular immune response to it. A few of the methods used to diagnose Johne's disease are listed:

1. Culture of the organism on Herrold's egg yolk agar containing mycobactin. Typical colonies of acid-fast bacteria are observed after prolonged incubation (12-16 weeks, growth after 20 weeks is not uncommon) on mycobactin-containing slants. Slow growth rate and mycobactin dependency are used to identify the organism.
2. Addition of enrichments like egg yolk and mycobactin plus antimicrobials renders the Middlebrook 7H12 medium in the BACTEC system (used for diagnosis of *M. tuberculosis*) more selective. At the University of Wisconsin, Dr. Collins has been successful at using the BACTEC system to detect this organism with better sensitivity and in a shorter time than conventional culture.
3. IDEXX Laboratories, Inc. has developed a gene probe kit to detect the insertion sequence IS900, unique to Map. This kit allows you to take a fecal sample

through DNA extraction and PCR amplification to make a determination in only three days of whether Map is in a clinical sample. Presently the kit lacks analytical sensitivity, however, requiring greater than 10,000 Map/gm of sample to trigger a positive test.

4. CSL Limited in Australia and IDEXX Laboratories, Inc. in the U.S. market an ELISA kit for detection of serum antibodies to Map. The specificity of this assay in cattle is consistently > 99%. The test is made more specific by absorption of serum samples with antigens from a common environmental mycobacterial species, *M. phlei*, prior to doing the assay.
5. The newest of the techniques for diagnosis of Map are based on measurement of gamma interferon released by peripheral blood leukocytes after exposure to mycobacterial antigens. Early reports on the evaluation of this test for cellular immunity to Map are promising but only a small number of studies have been reported.

Given that we have more diagnostic power and tests of comparable accuracy for Johne's disease as compared to bovine tuberculosis and brucellosis, there is no reason why we cannot control this disease if we have the will to do so.

Is CD a foodborne disease? Can Map pass the pasteurization hurdle and wind up in our milk supply or in other diary products like yogurt or cheese? Experts agree that this organism disseminates to the udder and is excreted directly into the milk of infected cows, most likely inside leukocytes. Studies from Ohio State University, the University of Pennsylvania, and England all agree on this issue; and that it happens before the animal shows signs of diarrhea and weight loss. It is also possible that organisms in the manure can contaminate milk. The question is whether Map in raw milk is sufficiently heat-resistant to withstand pasteurization (72^{o} C for 15 sec). Unfortunately, in recent debates on this controversial subject there are differing results. Four studies indicate that Map is capable of surviving pasteurization, and two studies say it is not. It was found in pasteurized retail milk in England in 1996 and in late 1998 Irish workers claimed to have reproduced this finding. These studies have all involved fluid milk and no studies have been done on other dairy products, such as cheese, yogurt, ice cream, etc.

Thermal tolerance data indicate that this organism is more heat resistant than other bacteria that pasteurization is designed to kill such as *Coxiella burnettii* and *Mycobacterium bovis*. Dr. Collins believes it is indisputable that Map, and its cousin *M. avium*, are more thermal tolerant than these other pathogens. A more pertinent question is "how many Map occur in raw milk?" We don't know the answer. It is probably quite variable. Given its thermal tolerance, whether any Map survive pasteurization is a direct function of how many are in raw milk. If, in fact, Map survives pasteurization, it would suggest that children would have a higher risk of exposure by virtue of their rate of milk consumption as compared to adults.

There exists yet another potential source of human infection. When cows conclude their natural productive life, or when they develop a disease like Johne's disease and cease to be profitable for milking, they are sent for slaughter. Usually they pass inspection on the hoof and in the slaughterhouse but they do not become Grade A meat products. Instead, they are made into ground beef. As illustrated with the experimental infection data, due to the disseminated nature of Map infection, all parts of the carcass have anti-mortem contamination. Post-mortem contamination, i.e. fecal contamination of the carcass in the slaughterhouse, is another possibility, just as it is with *E. coli* 0157:H7. Grinding beef products thoroughly mixes contaminants into the product and creates a very nice milieu in which this organism can survive. Killing curves for Map in ground beef have yet to be determined, but it may be a protected environment for the organism and, since many of us like to eat ground beef cooked medium to rare, it is possible that the organism could survive cooking.

Water contamination is another possible means of human exposure to Map. Contamination of surface waters from agricultural sources is a common problem. Surface waters feed the domestic water supplies of many communities in the U.S. Map is not likely to be killed by anything we do with domestic water treatment, including filtration or chlorination based on recent studies on its closest relative *M. avium*.

Similarities between Johne's disease and Crohn's disease:

1. Both diseases begin early in life.
2. Clinical manifestations of both diseases begin after sexual maturity. There is some evidence to suggest that hormonal cycling, associated with calving and lactation, plays a role.
3. Both diseases cluster in families. For humans this is interpreted to mean there's a genetic linkage. The same familial clustering occurs in Johne's disease but it is interpreted as a function of the high frequency of transmission between a cow and her calf.
4. The target site of disease is the ileum for both Johne's disease and Crohn's disease.
5. The host response in both diseases, as seen by histopathology, is quite similar.
6. Clinical symptoms are essentially identical.

Differences between Johne's disease and Crohn's disease:

1. Traditionally, Johne's disease has not been considered to be segmental, while skip lesions are described for CD. However, the veterinary community may be changing their view on this. As we investigate cases of Johne's disease more extensively, we find a discontinuous distribution of Map in the gut and regional lymph nodes of infected animals.
2. Acid-fast bacteria are not seen in tissues from CD patients while they can be found in most cases of Johne's disease, if we look hard enough.
3. Animals do not have the manifestations of bowel stenosis and perforation observed in people with CD. However, animals with Johne's disease are not typically treated. Rather, they are slaughtered, so we do not know what the clinical progression of Johne's disease would be if we treated for a prolonged time with antibiotics or immunosuppressive therapies.

Dr. Collins discussed some unpublished clinical data. The same techniques used in animals for the diagnosis or detection of Map were applied in people with Crohn's, ulcerative colitis, or other problems. A large collaborative study, taking place jointly in Denmark and in Wisconsin involving surgical patients as well as outpatients with CD and ulcerative colitis is ongoing. The Crohn's and Colitis Foundation support this work. Some preliminary data from this study were presented.

With Wisconsin patients, an average of 5.5 tissues were sampled per patient, including multiple intestinal sections and regional lymph nodes. These tissues were homogenized, coded, and tested blind by Dr. Gorm Lisby in Copenhagen. Dr. Lisby performed a nested primer PCR for the IS900 sequence of Map. Only after those results were returned, was the code broken. We found 1 of 21 controls, 12 of 31 Crohn's patients, and 13 of 35 ulcerative colitis patients tested positive for the IS900 sequence. Not all tissue samples from a given patient tested positive. Detecting Map was a low frequency event, even within given PCR-positive patients. When ELISA was used to measure serum antibodies (IgG) to Map, they found a significantly higher rate of positives in the Crohn's patients than in the ulcerative colitis patients or controls. The third diagnostic test involved gamma-interferon assay as an indicator of cellular immune response to Map. The results of this assay thus far are inconclusive. Attempts to culture Map from the resected intestinal tissues using the modified BACTEC system have not been successful to date.

He discussed antimycobacterial drug treatments. Therapy of Crohn's disease with antimycobacterial drugs has used first line anti-tuberculosis drugs rifampin, ethambutol, isoniazid, and pyrazinamide. Only recently have clinical trials with antibiotics such as the quinolones or macrolides been attempted. Determining the MIC values for these antimicrobials against four human-origin and one animal-origin type strain of Map has shown that these organisms are mostly resistant to first line TB drugs. Based on this *in vitro* evidence, it is unlikely that TB drugs alone would have much effect. Several things were learned from the pilot study on drug susceptibility of Map:

1. The ATCC type strain of Map is a particularly poor model for determining antimicrobial activities. Clinical isolates should be used.
2. Traditional TB drugs are not efficacious against Map. These drugs did not show even the slightest degree of growth suppression.
3. Susceptibility testing methods must be adapted to deal with a species that grows more slowly than the so-called "slow growing mycobacterial pathogens".
4. Clarithromycin and sparfloxacin did show promise.
5. Studies should be expanded to look at combinations of drugs and to look at their efficacy against intracellular organisms

Dr. Collins summed up be stating that he does not know whether Map causes Crohn's disease or any other human ailment for certain, but he thinks that there is ample evidence to cause concern. A conservative response to this situation is warranted because the magnitude of the problem, otherwise, is enormous.

Question: What is your experience with antimicrobial or immunosuppressive treatments for infected animals?

Answer: It's really never been done. The longest they've ever attempted treatment is about a month. Clotrimazol and a few other drugs have been used, but nothing has been used for the prolonged 6, 9, or 18 months duration typical of human treatment regimens. These animals weigh 1200-1500 lbs. and the cost of drugs is just staggering.

Question: What happens if you give steroids? This is a wonderful opportunity to study the effects of drugs that we give to humans.

Answer: I totally agree. Since 1992, to the best of my ability to determine from public records, USDA has only awarded 3 grants of significant size (>$100,000) for research on Map. Meaning, even the veterinary research community is not doing much work on this pathogen. Until other agencies support research and other investigators are drawn to study this organism, I don't think you will get the kind of data you are looking for.

Question: I'm concerned about the MIC values you showed. They seemed significantly out of line with what's been reported for *M. avium* in general.

Answer: This organism requires some manipulation of the drug susceptibility testing methodology due to its slow growth rate. I think the data are best considered relative to the different drugs within a trial, not as compared to MICs reported for other organisms by other studies. For an organism with a generation time of 48 hours, you can't do the assays the same way.

Question: What is known of the international distribution of Johne's disease?

Answer: In terms of presence or absence, there's essentially no country or geographical area free of this infection. Prevalence rates differ, but those are heavily influenced by the capacity of

the diagnostic centers in those countries to find the infection. But it's been reported with a worldwide distribution. Two pockets where we think the disease is absent are Western Australia and possibly Sweden although in the latter country only cattle have been surveyed.

Question: How would you explain the presence of Crohn's disease in Sweden?

Answer:I can not offer an explanation.

Dr. John Hermon-Taylor - Data Supporting Map as a Causative Agent of Crohn's Disease

Dr. Hermon-Taylor reiterated some of the points raised by Dr. Collins regarding Johne's disease in animals particularly the broad range of histopathological types from pluribacillary to paucimicrobial like leprosy in humans. He also noted that in cattle infected with Map, fecal culture is falsely negative in about 20% of cases, and in infected sheep in 60-80% of cases. Laboratory culture is not a reliable method of detecting Map in animal, food, and environmental or human clinical samples. However, the Map genome contains 14-16 copies of the 1451bp DNA insertion element termed IS900 that is uniquely specific for this organism. In recent years IS900 has provided a convenient target for DNA-based detection systems.

DNA fingerprinting of 740 isolates of culturable Map from infected animals and humans around the world carried out by Dr Ivo Pavlik in the Veterinary Research Institute, Czech Republic, has so far identified 23 different genotypes. A multiplex PCR typing system for MAP is applicable to culturable as well as unculturable forms, supports the view that different strains of Map have emerged with individual host preferences for bovines, ovines, and for humans.

Dr. Hermon-Taylor then presented a detailed description of the occurrence of CD in the UK. Western Europe has a high incidence of the disease estimated conservatively to be 280,000 rising at the rate of 23,800 per year. He pointed out that certain "hot spots" occur and that they are coincident with areas that use water supplies that can be contaminated by agricultural run-off. Map could be periodically detected with the IS900 probe in these waters. He made the case for the possible spread of Map by contaminated waters or their aerosols and pointed out that standard water disinfection methods would not eliminate Map. From late 1990 to mid 1994 he carried out an extensive survey of MAP in retail whole pasteurized cows' milk widely obtained throughout central and southern England and South Wales. With peaks in January, February, March and September, October, November, an overall 7% of retail milk cartons tested positive. Some liquid cultures were seen to contain visible acid fast mycobacteria after 6 weeks of incubation and tested strongly positive with IS900 PCR. He concluded that in the U.K., the risk that MAP is being conveyed to the human population in retail milk supplies is very high. Recently, Dr. Irene Grant in the Department of Food Science, University of Belfast, Northern Ireland, has developed an immunomagnetic capture method for MAP applicable to milk sample processing. Using this, together with BACTEC liquid culture and IS900 PCR, she recently tested 31 cartons of pasteurized milk from 16 pasteurization plants and found 6 (19%) to be positive.

Dr. Hermon-Taylor cited 6 studies each reporting residual culturability of Map at least once following exposure of spiked milk to experimental pasteurization at 65^{o}C for 30 minutes or 72^{o}C for 15 seconds, and two other studies which reported no culturable organisms remaining. He invoked the "viable but non-culturable" state to explain why individuals may be infected with food or environmental samples that fail to demonstrate the presence of "culturable" organisms. Such a state has been demonstrated in other bacterial species such as *Vibrio cholerae*. In general, the problem of identification of Map in samples can be attributed to the difficulty of culturing this organism. A recent study by Dr. Ivo Pavlik in the Czech Republic cultured 2 strains of Map from fresh tissues of 39 CD patients. The detection rate of laboratory culture of Map in Crohn's disease has risen to about 30% when IS900 PCR has been applied to long term cultures. Recent work by Dr. Saleh Naser and colleagues from the University of Central Florida, using an improved liquid

culture incubated for about 10 weeks followed by IS900 PCR, has so far detected Map in 6 of 7 Crohn's disease patients and none of 10 non-IBD controls.

Tissue preparation may facilitate the identification of Map by IS900 PCR applied directly to DNA extracts of Crohn's disease tissue. Using fresh frozen tissues from sheep with paucimicrobial Map and humans with Crohn's disease, it was shown that treatment with 6M guanidine HCl at 50^{o}C or prolonged incubation in SDS with proteinase K, are not sufficient for Map. Reliable access to Map DNA in both sheep and human samples required dissolution of the sample in SDS proteinase K followed by mechanical disruption for 45 seconds using the Hybaid ribolyser system followed by nested PCR for IS900.

Dr. Hermon-Taylor then summarized what is known about immunological detection of Map in humans. A number of protein antigens from Map have been identified by researchers and are recognized by a significantly greater proportion of CD patients than controls. Among these are two antigens of 35K and 36K molecular weight (see Dr. El-Zaatari's transcript). In immunoblots, IgG recognition of either or both of these proteins was 92% by CD sera and 25% in controls ($p<0.01$); recognition of both proteins was seen in 75% of CD and none of the control sera. The Map specific insertion element IS900 that is the target of PCR diagnosis encodes a 43kDa protein on its positive strand. Studies using RT-PCR have identified p43 mRNA expression in association with inflammatory bowel disease in humans. A preliminary study suggested the presence of an epitope within the carboxy terminal portion of p43 that is recognized by CD sera. This has subsequently been confirmed in a blinded study using sera from 104 CD patients and 104 age/sex matched controls. IgG binding was elevated ($p< 0.0001$) in Crohn's disease compared with control normal subjects. Recent outcome analyses have reported substantial remission in active Crohn's disease following treatment with combinations of rifabutin and clarithromycin or azithromycin, drugs known to have enhanced activity against *M. avium* complex. Controlled trials are needed.

Dr. Hermon-Taylor summarized his view of the pathogenesis of CD as follows. Some strains of Map are pathogenic for humans and cause chronic granulomatous inflammation of the intestine. We are unlikely to make satisfactory progress in elucidating the causation of CD and the role of Map if we think CD is like tuberculosis. Although we do not yet know its pathogenic mechanisms, these are unlikely to involve the release of damaging microbial toxins, or of molecules which are directly granulomagenic. Map is present in low abundance within a critical population of target cells such as macrophages. It is much more likely to cause an intermittent immune disregulation within the gut wall and elsewhere, in humans with an inherited or acquired susceptibility. Together with an accompanying defect in mucosal integrity, this results in a chronic inflammatory and allergic response to bacteria and other constituents present in the fecal stream.

Clinical improvement can be achieved by suppressing or modulating the immune response itself or by reducing the intensity of the allergic component by diet. Some clinical improvement can also come from the use of general antimicrobial agents. However, without killing the causative agent, such therapeutic approaches are unlikely to achieve lasting resolution of the disease. New drugs and immunotherapeutics active against MAP are needed. By preventing infection, however, we can prevent CD in the first place. Strategies aimed at controlling Map in domestic food animals and water supplies may help in this regard.

Question: I'd like to explore the cell-mediated immune response. It's always bothered me that we haven't been able to detect a CMI reproducibly in CD. How could mycobacteria alter antigen presentation to deregulate the normal dendritic function. John, could you expound on how you think engulfing Map with M cell macrophages could deregulate the immune response, and maybe we could hear what might happen in leprosy with APC activity?

Answer: This is entirely putative but it's consistent with what we know and what we see. The model that I was sketching is not one like tuberculosis. It's one in which this organism, in a

phenotype that we badly need to identify, becomes taken up by macrophages and I would guess it exists free in the cytoplasm. It's not in the bacillary form. It can just sit there and do nothing, like an inert mitochondrion. But if the relationship between the two is perturbed, and this can be a perturbation that is made more likely on the basis of an inherited or acquired susceptibility – a susceptibility which includes psychological stress factors known to perturb the immune system. Then, a disruption of the normal immunoregulatory functions of APC's and macrophages can occur, which by analogy with other tissues leads to imperfections in the function of the overlying epithelial layer. An overreaction to normal flora or food results in an inflammatory process within the bowel wall. The best mimic of this is the IL10 knockout mice or the cadherin gene knockouts. I think the way that fits with the evidence that Dr. Bayless and others were discussing is that the immunologic reaction that characterizes the major portion of the disease process is not against MAP itself. It's against these other things. So if we apply non-specific inflammatory influences, we can temporally control the disease, the same with reducing the allergic component, the same with reducing the ambient microbiological flora, and that would fit. Of course, the signals that allow the organism and the cell to communicate are not known.

Comment: There is compelling data in animal models, that normal bacteria provide the antigenic drive, but if a mycobacteria could alter the host immune response to those bacteria, I could see how a very paucibacillary form of disease could cause inflammation. It's always been my personal hang-up with the whole mycobacterial theory, that how does an extremely paucibacillary, or paucimicrobial form of infection, end up causing the disease? We have to wrestle with that, I believe, in order to understand how a small infection might cause chronic inflammation.

Comment: Tubercular leprosy is a classical example. The paucibacillary form of the disease can't really be diagnosed on the presence of organisms. You look at the pathogenesis as manifested nerve damage, and so on. It happens to be granulomatous and it happens to have a very strong cellular immune response so it's easy to detect the presence of an invisible antigen immunologically, but you can't usually find organisms in those patients. Obviously it's possible, I think the difficulty here is that we can't find the organisms and we can't see a good T cell immune response, so we're sort of stuck without a good model. We have to create a new model that combines the lack of organisms, or very low number of organisms, or modified organisms, with a somewhat unusual cellular immune response. We have to think very creatively to come up with a new model.

Comment: There's no doubt that you can induce a cross-reacting self-perpetuating autoimmune response due to molecular mimicry from an inducing agent. The inducing agent goes away but the immune response continues. But you still should have an immune response against the original inducing antigen in that scenario.

Dr. Herbert Van Kruiningen - Data That Do Not Support Map as a Causative Agent of Crohn's Disease

Dr. Van Kruiningen summarized the evidence that argues against the hypothesis that CD is caused by Map. He first pointed out areas where CD and Johne's disease are similar:

1. There is affliction of the small and large intestine.
2. There appears to be a long incubation period and prolonged course.
3. There are non-caseating tuberculoid granulomas in some species of animals with Johne's disease and certainly in Crohn's disease.
4. There is lymphocytic or granulomatous lymphangitis that is rare in Johne's disease but certainly is a very important feature of Crohn's disease.
5. There is focal ulceration of the Peyer's patches or the intestinal mucosa. This is rare in Johne's disease.

Dr. Van Kruiningen then went on to summarize his view that Map is not the causative agent of CD.

1. He described many pathologic features of CD that are never observed in animals with Johne's disease, such as rigidity of the diseased bowel, segmental stenosis with dilated loops of intestine proximal, transmural inflammation, fistulas, loop to loop adhesions, perforations and abscesses. Ulcerations are extensive in CD and bleeding occurs, but such is not the case in Johne's disease. Surgical resection is never called for in Johne's disease, even in animals that are kept for up to 10 years. The extra intestinal manifestations of CD do not occur in animals with Johne's disease.
2. Since 1982, there have been only seven or eight recoveries of Map from CD patients by microbiological methods despite numerous attempts. Earlier, Van Patter made 1762 culture attempts on tissues from 43 patients and they were all negative for Map.
3. Many attempts have been made to transmit CD disease to animals employing diseased tissue as inoculum and using various routes of inoculation. These animals have included guinea pigs, rabbits, chickens, cats, rats, and goats. None developed gross or microscopic evidence of CD. When, in the 1980's Van Kruiningen et al administered Map to infant pygmy goats, lesions of Johne's disease resulted; lesions of CD did not. Although Map has been given experimentally to numerous species of animals, which were then followed for months or years, CD has never resulted.
4. CD patients do not recognize Map with their immune system. Conclusions from a number of studies were quoted that failed to identify an antibody or cell mediated immune response to a number of diverse Map antigens.
5. Immunocytochemistry methods have been applied to the resected tissues of patients with CD in an attempt to demonstrate antigens of Map. These have been consistently negative.
6. Eight most recent PCR studies conducted since 1995 have uniformly failed to demonstrate evidence of Map in diseased tissues.
7. Finally, although Johne's disease is common in farm animals, and infected animals shed Map in large numbers, no record of zoonotic transmission has been recorded.

Dr. Gilla Kaplan - Is There a Middle Ground?

Dr. Kaplan attempted to define a middle ground between the two views concerning Map as a causative agent of CD. She pointed that there is more similarity between Crohn's Disease and leprosy than was appreciated previously. In both diseases there is the difficulty or inability to culture the organism. In *M. leprae* infection, especially in the case of paucibacillary tuberculoid leprosy patients, it is not only impossible to isolate and culture the organisms *in vitro*, but there are major difficulties in identifying the organisms in the lesions morphologically by staining and PCR. In tuberculoid leprosy patients with a well-developed granulomatous response, organisms are seldom found and definitely not cultured from biopsy specimens. Therefore, not being able to culture *Mycobacterium* from CD does not argue against them as an etiologic agent.

The inability to find or identify granulomas in CD is also similar to leprosy. Lepromatous leprosy patients have multibacillary lesions containing readily apparent mycobacteria (shown by acid-fast staining and PCR) and, unquestionably, have a mycobacterial infection. However, these patients do not have fully differentiated granulomas. In these patients, it is difficult to detect any mycobacterial-specific cellular immune response. There is little, if any, thymidine incorporation in stimulated lymphocytes or gamma interferon production in response to mycobacterial antigens *in vitro*. Thus, the absence of a specific cellular immune response against mycobacteria in CD may be analogous to the lack of a cellular immune response in lepromatous leprosy.

The observation that an exacerbation of Crohn's is not seen in HIV infected individuals or in patients immunosuppressed by steroid therapy, also does not argue against a mycobacterial infection. This also is the case with leprosy. HIV infection has not been shown to exacerbate leprosy, nor has co-infection with HIV shown any major impact on the number of leprosy patients or the number of organisms found in the lesions of patients, even in advanced AIDS. Leprosy patients are very often treated with steroids and there's no real indication that steroid treatment increases the bacterial load. Steroid treatment reduces the inflammation, lowers cytokine production and provides some symptomatic improvement, but there seems to be no relationship between the use of immune suppressive drugs and the growth of the organism.

Another interesting observation in leprosy is the fact that long after leprosy patients have been cured bacteriologically (following multidrug therapy), many of the symptoms associated with the reactional states of leprosy and the chronic inflammation persist, even in the absence of any viable organisms or any detectable antigen. Something has been triggered early on by an infectious agent that induces a state of immune sensitization, which persists, even in the absence of detectable organisms or antigens.

Dr. Kaplan also pointed out that the identity of a putative antigen that drives the host immune response is not known in CD. Much work has been done to find T cell responses specific for mycobacterial protein antigens. These have not been found, and it has been used as an argument against a mycobacterial etiology. Perhaps, we are not looking for the immune response to the appropriate antigens and in the appropriate subset of lymphoid cells? It is clear that mycobacterial antigens, such as those identified from *M. tuberculosis* and *M. leprae* could easily be lipids or carbohydrates. The responding cells could be CD1-restricted lymphoid cells. These should be considered for CD. T cells from tuberculoid leprosy patients do recognize protein antigens very efficiently. Maybe Map infection is more like *M. avium* infection, where detection of a cellular immune response in humans is much more difficult. Perhaps somewhere between *M. leprae* and *M. avium* lies the truth.

In summary, looking at the problems raised here from the perspective of what is known about the immune response to other mycobacteria, Dr. Kaplan did not think there was enough evidence to prove or disprove an association between mycobacterial infection and Crohn's disease. If we assume that there is an infection, but that the infection is not like tuberculosis but more like leprosy or *M. avium*, it could lead to more creative research approaches to identify a causative agent(s), the mechanisms of pathogenesis and potential anti-microbial treatments.

Dr. Norman Pace - Human Pathogenesis and Microbial Ecosystems Analysis

Dr. Pace presented himself as an environmental microbial biologist or microbial ecologist, who looks at this and other types of disease as ecological problems. He pointed out that environmental microbiology is an important area, but that we know relatively little about naturally occurring microbial populations. This is because we haven't been able to study them until quite recently because we haven't been able to cultivate them. Of the organisms in the environment, we can't cultivate 99.99% of them. He felt that the pathogens or commensals we have identified represent only a few percent of those that exist.

Evolutionary relationships between organisms can be estimated based on sequence divergence of conserved genes like ribosomal RNA genes. This technology gives one the ability to define the organisms present in any particular environment. Definitions of organisms in the context of sequence are very different from the traditional definitions of organisms in the context of culturability and physiological traits. There is no question about the identification of organisms in the context of sequences. Organisms with the same or similar ribosomal RNA will in general have similar physiological properties. Using these molecular techniques, one can identify the components of microbial communities.

This technology needs to be applied to CD. By designing different probes, one can look broadly at groups of organisms or specifically at species or strains. Dr. Pace's group has recently begun to use this technology to look at non-bacterial chronic prostatitis. It is called non-bacterial prostatitis because there's nothing consistently cultivated from expressed prostatic secretions, but when patients are treated with antibiotics, they generally tend to improve, as with CD. They have identified *Corynebacterium* species and sub-species frequently associated with prostatitis. These organisms were found in both normal and in diseased secretions, but the suite of organisms that are recovered are not the same. So, in CD, it is quite likely that different suites of organisms will be enriched throughout the course of the disease. The organisms that one sees may be influenced by the genetics of the individual patient.

It's probably a mistake to limit the search to a specific etiology. The possibility exits for a group of organisms that may or may not have a specific property. Appropriate ribosomal RNA sequences should provide an accurate description of that group. This technology really defines the question of Crohn's disease - what kinds of organisms are associated with the syndrome? It's probably going to be suite of organisms, which will vary with the patient, and there probably will be a genetic component, and there certainly will be an immune system component. The important point is that it may be over simplistic to talk about the single, specific pathogen. It may be that the disease is the response to a community of organisms rather than a particular organism.

Dr. David Relman - From Sequence to Causation

Dr. Relman reviewed the kinds of non-cultivation dependent or sequence-based methods that we have currently available for identifying organisms that have resisted characterization. He talked about their relative advantages and limitations as they might be applied to Crohn's disease.

He pointed out that there are two basic issues and problems that we face. The first is, the association of a particular sequence or group of sequences with the disease, and to decide whether or not the inferred organisms are actually causing disease, or are bystanders. The second issue is the non-specific nature of the pathology, the paucibacillary disease where you can't find an organism, you can't grow an organism, and you can't always detect specific sequences by PCR.

Dr. Relman stated that the detection of ribosomal DNA is the most useful method available at the present time, based upon sequence databases. Lots of ribosomal DNA sequences are known and they are reliable for establishing phylogenes. The selectivity can be as wide or as specific as you would like it to be and will put you in the right part of the phylogenetic tree. From there, one can choose a different genomic target for a more refined comparison. One might be able to select sequences that have not only phylogenetic value but also encode antigens that could be expressed, and against which one could look for an immune response.

The disadvantage of these methods is due to their exquisite sensitivity and specificity; they are susceptible to problems of contamination, both extrinsic to the whole reaction, as well as intrinsic to the tissue - that you select areas not associated with pathology.

He drew on his characterization of the flora of the subgingival crevice for illustration of the kinds of data one can obtain. He had 56 phenotypically distinguishable isolates that could be cultured versus 264 that were detected from a directly amplified clone library. Of the clones, about 30% are different from anything known in the database at the level that one might attribute to species or genus definition. We simply don't really know to whom they are related in a more precise way. He stressed that this is not an infallible method for identifying all organisms present, but that there are constraints based upon the reagents used. It is important to choose supplemental or accessory reagents that will complement the sequences that are found in the initial screen.

Another technology is that of high-density arrays of DNA or RNA probes on a solid state surface, the DNA chip. The idea is to design an array that could sample and characterize ribosomal DNA molecules of many different species and then look at hybridization patterns by fluorescence. This is something that is being developed at Argonne National Labs and Lawrence Livermore Labs and a number of academic laboratories. This technology will provide quantitative data on up to tens of thousands of different ribosomal DNA sequences, simultaneously in an automated format. This is going to be technically much more feasible in the next few years than it has been to date.

Another method discussed by Dr. Relman was subtractive hybridization to look at the difference between one sample and another when there is a large amount of complexity in the background. He did not describe this in any real detail, but one can target any different molecule, any putative pathogen by using a broad subtractive method. The other advantage is that it incorporates a negative control as part of the experimental design. It becomes essential that one pick two well-matched tissues that ought to have the same background in order to have meaning in what one finds as the difference. The limitations here are that a product sequence, the difference product that one isolates after this procedure, may not necessarily allow you to identify the agent, at least at first glance. Dr. John Braun from UCLA is using this method to look at CD and he does have some very interesting products that he's been able to find in Crohn's tissue that are not found one centimeter away in the same patient. He's looked at some of these sequences and he talked about this at the Infectious Disease meetings just last month. The product sequence although extremely interesting and highly associated with CD does not necessarily give you a ready identification because it's a random piece of what looks to be a bacterial genome. But what bacterium is it? He doesn't know yet.

The third approach is looking at expression libraries. This was the method used to identify the hepatitis C virus. It also has advantages in terms of breadth of range. It also incorporates within its design the idea that the host has reacted against the product of the sequence that one pulls out. That's the basis for the screen. So it enhances the value of the sequence that one comes up with. The problem is that it's incredibly laborious as it's presently configured, although there may be some refined ways of speeding this up. It requires a humoral response, for which Crohn's disease may be somewhat lacking. It also generates false positives, if in fact your disease process involves some element of autoimmune sensitization. So you have antibodies, but they are directed against non-pathogen encoded molecules.

Where might microarrays fit into a pathogen discovery program where one doesn't have a sequence available? The idea is to display a large number of ribosomal DNA sequences on a chip, as well as broad range sequences to try to categorize and even identify a putative organism or group of organisms. Another idea is to look at the host as a reflection of disease, to use the complexity of information that's available now with a host oriented gene display for the purpose of identifying a pathogen. The idea here is that one can characterize the host gene expression response to infection by a particular pathogen or type of pathogen. One can then use that response to identify the type of organism that may be responsible for a disease of unknown etiology. Ron Davis and his co-workers showed that with a limited 1,000 gene array, there were some interesting, although somewhat nonspecific response elements that were either up or down-regulated in rheumatoid arthritis.

A question was raised about the complication of background in some of these tissues? One technical approach would be to use laser capsular microdissection. With this method, you can cut out a granuloma or even a single cell if you think it's a worthy target. You can also cut right through different levels of the mucosal wall, as well as different parts of a lymph node, and then use this tissue for broad range ribosomal DNA PCR.

The next issue is prognostic value of the sequence; e.g. does the sequence "respond" to treatment? Does it predict the later development of disease? In the case of Map, it would be nice to show that people who have the sequence at the expected sites who don't have disease go on

to develop disease at some point in the future. That has been done with Kaposi's Sarcoma-associated Herpes Virus (KSHV). The sequence ought to become less abundant after effective therapy, given the caveats discussed about whether or not steroids would constitute effective therapy and whether that would mean that you'd expect the sequence to diminish at all. But with therapy effective against the bug, there ought to be fewer copies of its genome after treatment. One would also predict that it ought to become more abundant again as the disease relapses or recurs.

Plausibility of an association is posed with some trepidation even though genotype ought to correlate with phenotype. If a sequence is identified that is not at all closely related to anything known, how consistent will its association with CD be? That's something that we don't really know. This is an area where the idea of a mycobacterium being responsible for CD is an attractive one.

inally, what about the direct correlation of sequence with pathology? One ought to be able to show that the specific sequence hybridizes to areas of pathology where one would expect the organism to be. Likewise, one expects that antibodies directed against true microbe-encoded antigens should locate areas of pathology. And finally, sequence ought to be focal using some of these microdissection techniques. How well can we predict where this organism ought to be? Dr. Relman was not all that confident about an answer. There are issues of intestinal versus extra-intestinal, location along the intestinal tract, as well as through the depth of the intestinal wall. There is the problem of the stage of the disease and which patients and which sites one might expect to be appropriate targets for these studies based upon their known proclivity towards later development of disease. For example, surgeons will say that in areas of re-anastomosis following resection that there is a higher likelihood of disease recurrence in CD. Collaborations between pathologists, surgeons, microbiologists and molecular biologists are needed to design useful studies.

Dr. Relman found the possible association between an infectious agent and CD to be intriguing. None of the implied conclusions from studies of Map exclude the possibility of multiple agents, other agents, and perhaps a completely different causation for a certain subset of patients. Dr. Relman argued that what we really need is to step back and define the microbial community associated with areas of pathology. For that, some of the newer methods should be applied. A good repository of samples is also needed. That is something that bespeaks the need for collaborations and consortia that need to be fostered. New technology that we didn't have several years ago will be even further improved in the next few years. He felt that we have to be incredibly rigorous and careful about what we say regarding the meaning of an association of this disease with a particular organism before concluding that there is an etiology.

Question: How would you like tissue stored?

Answer: That's a very good question. I can tell you that frozen tissue is nice to have. We would prefer frozen tissue in the absence of anything else added to a tube, such as OCT or other preservatives. One should use tubes that have been already documented to be free of DNA, RNA, etc. The issue of what type of tissue is another aspect. In the case of paraffin embedded tissue, then you're talking about a whole other range of issues such as age, nature of fixation, and other variables. It would be something that should be discussed prior to initiating studies. We've been able to amplify certain specific sequences very easily out of fixed tissue. But getting large chunks of sequence using broad range primers from fixed tissue is difficult, you're restricted to just 200-300 base pair fragments with typical fixation methods.

Comment: Dr. Pace commented that soon after taking the tissue, he drops it into 70% ethanol, 10% phenol. It is then considered formally disinfected, it can be shipped around quite freely, and you can get nucleic acids out of it very cleanly.

Dr. Rob Fleishman - Use of Sequencing, Bioinformatics, and Microarray Technologies to Identify Pathogens

Dr. Fleishman described the current efforts underway to sequence the entire genomes from pathogenic microorganisms and the wealth of information that can come from that data. The *Mycobacterium* community has benefited from this effort. Recently published by Stuart Cole, the H37 RV strain *M. tuberculosis* was completed by the Sanger Center. The Institute for Genomic Research (TIGR) is nearing completion of a clinical isolate, CDC 1551 referred to as the Oskosh strain, a highly virulent one. TIGR is also sequencing the *M. avium* genome from strain 104 that could be the most relevant to Map. We can think about how to leverage this sequence into learning more about Map. TIGR is about to release the first three-X coverage through their FTP sites, so that sequence will shortly be available to the community. The data will contain assemblies of about 30 to 35,000 sequence fragments. The Sanger Center is currently completing the *M. leprae* sequence, and *M. smegmatis*, BCG, and *M. bovis* may be done in the future. Improvements in the machines used for sequencing now allow one machine to generate 800 sequence fragments a day over a 24 hour period in an unattended mode. This greatly increases capacity for doing more sequences and will eventually bring down the cost as well.

There are three major areas that one would hope to benefit from sequence availability. One would be in the area of detection - being able to detect different strains, type strains, relationships of various clinical isolates, and perhaps providing more sensitive tests for diagnosis. Clearly, in the area of therapy, rational drug design would be possible after having the sequence available. The third area is improved vaccines. It is not certain that a vaccine strategy would be appropriate for Crohn's disease, but the dairy industry certainly would benefit from an effective vaccine against Johne’s disease. The genome sequence would provide information on developing attenuated strains, developing potential targets as subunit vaccines, as well as the potential for DNA vaccines.

When the whole genome sequence is available, it goes through a bioinformatics stage where all the interesting features of the genome are identified. Open reading frames and likely functions for genes are assigned based on sequence homologies to known genes. Another group of genes can be classified as hypothetical because the sequence match is not so close. For TB, about another 40% of the genome has no database matches.

Genome arrays on chips is really important technology. When we think about how we can leverage sequence information to looking at other genomes, we are primarily interested in using this for expression types of assays. An *M. avium* array would be particularly valuable for studies of paratuberculosis looking at gene expression and for comparative genomics in terms of what open reading frames or what elements of the genome are present in one and not present in the other. One can take two different strains, grow them under the same conditions, or perhaps the same strain grown under different conditions. The RNA is purified, it's labeled with two different fluorescent probes. The probes are then mixed and interrogated through hybridization with the microarray slide. Based on the amount of fluorescence, one can determine the level of transcription of particular genes in a comparative fashion. Tools like this are going to be extremely valuable for leveraging genomic information, for looking at differences in gene expression under various stress conditions or disease states.

Dr. Balfour Sartor - Possibility of Other Bacteria Triggering the Inflammatory Response in Crohn’s Disease

Dr. Sartor began his remarks by pointing out that there are a number of microbial agents that are being actively considered in the etiology of CD, in addition to Map. These include *Listeria*, measles virus, and non- *pylori*, *Helicobacter* species. In osteocolitis, there's some evidence of functionally altered luminal bacteria, and toxin producing *E. coli*. He proceeded to describe a number of genetically engineered animal models that may be useful in studies of CD.

Dr. Sartor stated that there is no compelling evidence in 1998 for a specific infectious agent being responsible for the majority of Crohn's disease or osteocolitis. He does believe that it remains a tenable hypothesis. He addressed two theories of CD, which basically incriminate normal bacteria, either due to a defective mucosal barrier leading to increased exposure to luminal antigens and dietary proteins that overwhelm the normal mucosal forces, or to an abnormal and overly aggressive host immune response to ubiquitous antigens.

In the first case, the concept is that in the lumen of the distal ileum and colon there are very high concentrations of predominantly anaerobic bacteria, bacterial cell wall polymers, chemotactic peptides and protein antigens capable of activating immune cells. In the setting of increased mucosal permeability either due to an intrinsic genetic determinant defect, or due to an environmental trigger, such as a transient non-specific infection, or exposure to non-steroidal anti-inflammatory drugs, the mucosal barrier is broached. This results in increased uptake of these bacterial products that activate the lamina propria macrophages and T lymphocytes to secrete pro-inflammatory cytokines and other soluble mediators that cause tissue damage. The more damage you get, particularly once you get ulceration, the more uptake of these bacteria occurs, perpetuating the inflammatory response. Thus, the normal luminal bacteria provide the constant antigenic stimulus that leads to these chronically relapsing conditions. Dr. Sartor stated that evidence to support this theory includes the fact that disease is located in areas of highest bacterial concentrations. The concentration of coliforms and anaerobic bacteria is about three logs higher in the distal ileum and the colon, the same area that's involved with CD. Inflammation improves when the luminal bacteria are decreased by broad-spectrum antibiotics, bowel rest, or bypass.

Probably the most compelling bit of evidence that bacteria are important are that germ-free rats and mice, those raised in a sterile environment have absolutely no evidence of chronic intestinal or systemic inflammation. A number of experiments were described using gene knockout mice that support a role for normal flora in intestinal inflammation in the right genetic background. For example, IL-10 knockout mice, in the absence of normal bacteria have no disease, and the wild type animals that do not have this genetic susceptibility can be colonized with the same bacteria that cause disease in the knockouts, but exhibit no inflammation. It's only when you have the confluence of the appropriate genetic susceptibility and the appropriate bacterial antigenic drive that you get chronic intestinal inflammation. In studies of colonization of HLA B27 transgenic rats with defined bacterial strains, Dr. Sartor reported that *Bacteroides vulgaris* was particularly able to induce inflammation while *E. coli* could not.

In closing, Dr. Sartor presented a hypothesis for CD, that chronic intestinal and systemic inflammation are due to an overly aggressive immune response to the normal resident luminal bacterial constituents mediated by Th1 lymphocytes and macrophages. Predisposing factors are genetic disregulation of immune responses and/or barrier function with onset triggered by environmental stimuli. In the gut there's a delicate balance between the constant antigenic stimulus provided by the normal bacteria and the mucosal protection that's mediated by a relatively impermeable mucosa and secretory antibody. So in the normal situation, there's a state of immunologic tolerance that prevents aggressive responses to these ubiquitous antigens. However, this balance can be perturbed by either genetic or environmental factors. Genetic factors are defects in immunoregulation and barrier function. Environmental triggers, including antibiotics that disrupt the balance of bacteria in the gut, diet, smoking, stress, anti-inflammatory use, and concurrent infection may alter the mucosal barrier. The concept that Map may serve as a perturbing antigen impacting on immunoregulation, is an intriguing one.

The basic research priorities generated by the Crohn's and Colitis Foundation about a year ago, included an understanding of the molecular genetic bases of host microbial interactions involved in CD. Dr. Sartor encouraged the NIH to investigate this very important area, not only for pathogens, but also in the way that genetically susceptible hosts respond to normal bacteria.

Question: Why do Crohn's disease and inflammatory disease occur in young people who are five years old or younger, while in other cases, some people do not develop the disease until they are 40 years old? Are those differences in genetic susceptibility, genes that will be turned on at some point?

Answer: Yes, I think that's a critical question, and one could suggest two possible answers. One is that the more genetically susceptible you are, the earlier in life you develop disease. The second is that the trigger factors are very important in determining when disease is initiated and reactivation of quiescent disease occurs. You don't develop disease until you have the environmental trigger that breaks the mucosal barrier, and initiates a response. Most people get well but the unlucky people that are genetically susceptible go on to chronic inflammation.

Dr. Fouad El-Zaatari - Investigations of the Humoral Immune Response in Crohn's Pathogenesis

Dr. El-Zaatari discussed the status of diagnosis of Map infection by serology. Bacterial antigens which react specifically with the sera of CD patients and which can serve as diagnostic for Map have been looked for for many years. Dr. El-Zaatari said the difficulty in identifying useful antigens could be attributed to the relative impurity of the antigens and mixtures of antigens studied and found to be non-specific. This is not surprising when one considers the many other mycobacterial species humans can be exposed to. It is necessary to work with purified antigens or preferably epitopes from these antigens in order to make diagnostic tests more specific.

Dr. El-Zaatari has identified 24 Map antigens by immunological screening of expression libraries with serum from Crohn's patients. These antigens ranged from 18 to 80 Kda in size. Two of these, a 35 Kda and a 36 Kda antigen, show promise as being diagnostic for CD.

P35 detected 100% of animals with clinically positive Johne's disease, 75% of sub-clinical animals, and was non-reactive in 15 normal animals. In sera from humans with CD, P35 detected 75% as positive; 10% of ulcerative colitis; 14% of controls; 25% of tuberculosis; and 0% of leprosy cases. This appears to be an interesting antigen that may be specific at the genus and species levels. It may be useful for detecting clinical and subclinical Johne's disease and CD. An epitope from this antigen is being pursued as a diagnostic.

P36 was reactive with 89% of the CD sera. However, it seemed less discriminating than P35 as 75% (6 of 8) TB sera were positive; 100% (10 of 10) leprosy sera; and 100% (7 of 7) sarcoidosis sera were positive. Only 15% of ulcerative colitis (N=27), and 7% of non-IBD sera (N=15) were positive. Animals with Johne's did not react with this molecule. This antigen reacted with antibody produced against eight different mycobacterial species. When P35 and P36 are used together, the specificity increased to 98%. The sensitivity of the pair is close to that for P35. The positive predictive value also is 98% and negative predictive value is 76% for the pair. Identification of reactive epitopes of these two antigens may make them even better diagnostics.

Question: Is it possible that these antigens are lipoproteins?

Answer: Yes. P35 is expressed on the surface and is secreted according to the Belgium group. I have no idea what their function is. They are useful as diagnostics. We just have to work more and identify more specific epitopes.

Dr. David Schauer - Development and Use of New Animal Models for IBD and Crohn's Disease

Dr. Schauer discussed the use of several knockout mouse models of inflammatory bowel disease. These include IL-2 deficient mice, IL-10 deficient mice, P glycoprotein deficient mouse

(the Mdr1A knockout), and T cell receptor (TCR) alpha-beta chain deficient mice. All of these animals develop inflammation in the intestine that is reminiscent in many ways of inflammatory bowel disease in people. The IL-2, IL-10, or TCR knockout mice when raised germ-free, do not develop disease.

His research group is interested in trying to establish a role of *Helicobacter* species in inflammatory bowel disease. There was a strong precedence with the recognition that *Helicobacter pylori* infection in the stomach could lead to chronic persisting gastritis, peptic ulcer and increase the risk for gastric cancer. Working with scientists at Dynax, immunodeficient SCID mice were reconstituted with a subset of CD4 positive T cells that bear high levels of CD45RB. This is an adoptive transfer model; the cells are taken out of BALB/C mice, put into SCID mice and the animals develop inflammatory bowel disease and wasting disease when they are also infected with *H. hepaticus*. The IBD has features that are reminiscent of Crohn's disease, which include transmural involvement of the bowel with granulomatous inflammation.

There is also some evidence that *H. hepaticus* infection can induce disease in IL-10 knockout mice. Animals that were not infected with *H. hepaticus* did not have significant inflammation at 16 weeks. Animals that were infected had a significant amount of inflammation in the cecum, colon, and rectum at 16 weeks. Animals that were controls and remained *H. hepaticus* free did not develop the disease. There does seem to be a relationship between infection and genetic background because in wild type mice that received *H. hepaticus* there was a lower incidence of disease.

Helicobacter infection does appear to be sufficient to trigger inflammatory bowel disease in susceptible mice. *H. hepaticus* can cause typhlocolitis, even in immunocompetent animals. That's different from the IBD that develops rapidly in these genetically engineered susceptible strains. Dr. Schauer believes that the normal flora ubiquitous in a species does not cause IBD in susceptible knockout mice. His research is focused on generating isogenic mutant strains of *H. hepaticus* deficient in single genes that can be tested in animal models for their ability to cause disease. They are in the process of characterizing these virulence determinants now.

Dr. Schauer stated that he believes that pathogens can initiate inflammatory bowel disease. He believes that the way they do this is by interaction with the epithelial cell. These bacteria provide a signal that is transduced to the epithelial cells which trigger inflammation and subsequent immune response. He believes that the chemokines released from inflammatory cells are key in pathogenesis of CD. It is important to investigate the organisms that may initiate CD if we have any hope of preventing disease. A wide net should be cast and not limit the search to *Mycobacterium* species, which have received a lot of the attention. He believes that *Helicobacter* should be sought as well. Ten or fifteen years ago, no one would have guessed that *H. pylori*, was the cause of ulcer disease in the stomach. It's now quite clear that it does cause ulceration and chronic inflammation in the stomach, and increases the risk of developing stomach cancer. He would like to determine in the next ten years whether that same relationship is true of *Helicobacter* and IBD.